

Research Note

In situ hybridization in *Vitis vinifera* L.

H. U. HAAS, H. BUDAHN and G. ALLEWELDT

Key words: *Vitis vinifera*, fluorescent *in situ* hybridization, nucleolus organizer regions, gene mapping.

Introduction: Cytological studies of metaphase chromosomes in *Vitis vinifera* L. were restricted to the determination of the chromosome number for a long time. Analyses at the chromosome level both in meiotic (HILPERT 1958, 1959) and somatic metaphase plates of grapevine were limited by the size of the chromosomes. Only few approaches to establish a karyotype by measuring homogeneously stained metaphase chromosomes have been made because of their small size (0.8–2.0 µm) and similar length (e.g. PATIL and PATIL 1992). For the differentiation chromosome-specific markers have to be developed. Apart from cytogenetic methods as C-, N-banding or silver staining *in situ* hybridization could be a useful tool for the characterization of chromosomes.

In 1969, a method for radioactive *in situ* hybridization was described. Since, it has been developed and in the 1980s it was more and more replaced by fluorescent *in situ* hybridization (FISH) which is widely used for the detection of DNA sequences now (Joos *et al.* 1994). As shown by LEITCH and HESLOP-HARRISON (1992) FISH is extremely valuable in studying karyotypes with small and similarly sized chromosomes in plants. Nevertheless, no studies in grapevine have been reported yet. For establishing this method in grapevine we intended to detect the satellite chromosomes by *in situ* hybridization. In the chromosomes the rRNA genes are located in the region proximal to the secondary constriction in form of several hundreds tandemly repeated units (MALUSZYNSKA and HESLOP-HARRISON 1993). As an rDNA probe we used pTA 71 from common wheat (GERLACH and BEDBROOK 1979).

In the present paper we demonstrate that fluorescent *in situ* hybridization can be used as a new, additional method for characterizing the chromosomes and for physical mapping of interesting DNA sequences (genes) on chromosomes of grapevine.

Materials and methods: Root tips from young *in vitro* plants of *Vitis vinifera* cvs Bacchus and Riesling were treated with icewater for 8–10 h, fixed in ethanol:glacial acetic acid (3:1) and stored in 70 % ethanol at -20 °C.

For metaphase preparations, a variation of the drop technique according to MARTIN *et al.* (1994) was used. Root tips were macerated for 90 min and the drops were used without filtration through a nylon mesh. Before hybridization, the slides were air-dried for 4 h.

The probe (pTA 71), containing the 5.8S-, 18S- and 26S-rRNA genes and the intergeneric spacer, was labelled with DIG-11-dUTP by random primed labelling after linearization (Boehringer, Mannheim, FRG). After ethanol precipitation, the labelled DNA was resuspended in the hybridization mixture containing 50 % (v/v) deionized formamide, 5 % (w/v) dextran sulfate, 0.1 % (w/v) sodium dodecyl sulfate (SDS) and 2 ng/µl sheared salmon sperm DNA in 2x SSC (0.3 M sodium chloride, 0.03 M sodium citrate). The probe was denatured at 75 °C for 6 min followed by a chilling step at -20 °C.

The slides were pretreated with 100 µg/ml RNase in 2x SSC (37 °C, 40 min), washed four times in 2x SSC, fixed in 4 % (w/v) paraformaldehyde in 2x SSC (37 °C, 15 min) and washed again four times in 2x SSC. After denaturation for 6 min at 75 °C and chilling in 70 % ethanol (-20 °C), 10 µl of hybridization buffer containing 100 ng denatured probe DNA were dropped on each chromosome preparation which was subsequently covered with a coverslip and sealed with Fixogum (Marabu, Tamm, FRG). The specimens were denatured for 10 min at 75 °C and following hybridization was carried out in a humid chamber at 37 °C for at least 12 h.

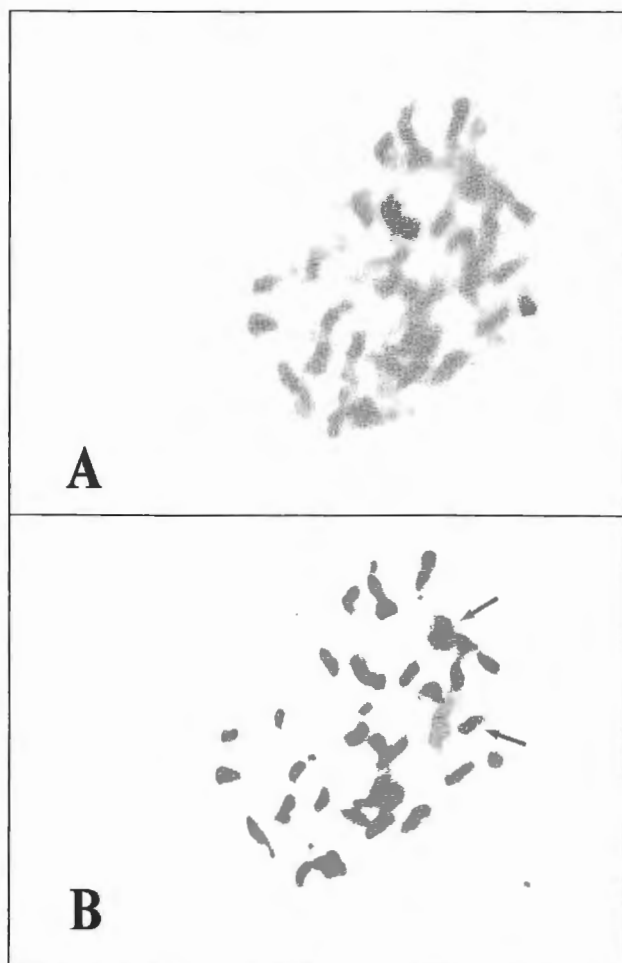
After removal of the coverslips, slides were washed twice in 50 % (v/v) formamide + 2x SSC (37 °C) and three times in 0.5x SSC (50 °C). Afterwards blocking solution (0.1 % (v/v) Tween 20 and 3 % (w/v) bovine serum albumin (BSA) in 4x SSC) was dropped onto the slides (30 µl each). Incubation lasted 30 min at 37 °C. 30 µl antibody solution containing 40 µg/ml anti-digoxigenin-fluorescein fab-fragments (Boehringer, Mannheim, FRG), 1 % (w/v) BSA and 0.1 % (v/v) Tween 20 in 4x SSC was added and the slides were incubated at 37 °C for 30 min. For increasing the hybridization signal 30 µl of 0.4 µg/ml anti-fluorescein mouse IgG1 (Boehringer, Mannheim, FRG) in 4x SSC were incubated on each slide for 30 min (37 °C). Anti-mouse-Ig-fluorescein (Boehringer, Mannheim, FRG) was applied (30 min, 37 °C). After each step samples were washed twice in 0.1 % Tween 20 + 4x SSC (37 °C). The slides were counterstained within a propidium iodide (PI) solution (200 ng/ml PI in 2x SSC) for 15 min and washed in 0.05 % Tween 20 in 1x SSC (1 min). Then, embedding in 2.3 % (w/v) DAPCO dissolved in 1 ml 0.2 M Tris-HCl (pH 8.0) and 9 ml Glycerol finished the procedure (BOEHRINGER 1992).

Metaphase spreads were examined with a Zeiss Axiovert 135 TV microscope with filter set 09 (long pass filter for PI- and FITC-fluorescence, 450–490/510/>520 nm) and filter set 15 (for PI-fluorescence, 546/580/>590 nm). Photographs were taken with different types of 400 ASA films and 8–30 sec exposure time.

Results and discussion: Following the evaluation of different FISH-methods (e.g. BOEHRINGER 1992, LICHTER and CREMER 1992, FUCHS *et al.* 1994, SCHMIDT *et al.* 1994) we started to develop a method for physical mapping of DNA sequences on chromosomes of grapevine.

After *in situ* hybridization two satellite chromosomes per metaphase could be observed in cells of Bacchus and Riesling. The rDNA-labelled chromosomes displayed yel-

low-green fluorescence signals near the secondary constriction. The stained chromosomes and the specific fluorescing signal could be observed with a Zeiss filter set 09 at the same time. With filter set 15 for propidium iodide-fluorescence the same chromosomes showed stronger red signals at the region near the secondary constriction. MALUSZYNSKA and HESLOP-HARRISON (1993) reported similar results for PI-stained chromosomes of *Brassica* species.



In situ hybridization of *Vitis vinifera* root tip metaphase chromosomes ($2x=38$) with DIG-labelled rDNA probe pTA71 (FITC) and Propidium iodide as a counterstain. – Bacchus: A) Propidium iodide fluorescence; B) *In situ* hybridization: two satellite chromosomes, each with fluorescence signal.

For the detection of well stained chromosomes with suitable fluorescence signals it is important to use good preparations of metaphase spreads. The drop technique method described above resulted in slide preparations with chromosomes fixed plain to the glassplate. The spreads could be observed without layers of cell compartments on the chromosomes. Changing the labelling and detection method from biotin-streptavidin-system to digoxigenin-system resulted in weaker background hybridization signals.

The number of the detected satellite chromosomes coincides with the results from former cytogenetic studies on homogeneously stained metaphase chromosomes (PATIL and JADHAV 1985; PATIL and PATIL 1992). For chromosome studies in *Vitis* species with varying numbers of satellite chromosomes (SHETTY 1959; SUDHARSAN RAJ and SEETHAIAH 1973) their determination using the FISH-technique as described above will be helpful to verify the correct number.

Fluorescence *in situ* hybridization is a useful tool for the detection of DNA loci on chromosomes of grapevine. Further progress in the development of adapted *in situ* hybridization methods will allow a comparison of genetic linkage maps (REISCH *et al.* 1994) with physical maps also in *Vitis*.

Acknowledgements: We gratefully acknowledge the help received by Dr. K. DÜRING, Federal Centre of Breeding Research, Quedlinburg and the provision of the rDNA-pTA71-probe by Dr. R. BRETTSCHEIDER, University of Hamburg.

- BOEHRINGER; 1992: Nonradioactive *in situ* Hybridization, Application Manual. Boehringer Mannheim GmbH Biochemica, Mannheim, Germany.
- FUCHS, J.; PICH, U.; MEISTER, A.; SCHUBERT, I.; 1994: Differentiation of field bean heterochromatin by *in situ* hybridization with a repeated FOK I sequence. *Chromosome Res.* **2**, 25-28.
- GERLACH, W. L.; BEDBROOK, J. R.; 1979: Cloning and characterization of ribosomal RNA genes from wheat and barley. *Nucleic Acids Res.* **7**, 1869-1885.
- HILPERT, G.; 1958: Untersuchungen an frühen meiotischen Stadien von *Vitis vinifera* L. *Vitis* **1**, 218-223.
- ; 1959: Untersuchungen am Pachytän von *Vitis vinifera* L. *Vitis* **2**, 79-83.
- JOOS, S.; FINK, T. M.; RÄTSCH, A.; LICHTER, P.; 1994: Mapping and chromosome analysis: the potential of fluorescence *in situ* hybridization. *J. Biotechnol.* **35**, 135-153.
- LEITCH, I. J.; HESLOP-HARRISON, J. S.; 1992: Physical mapping of the 18S-5.8S-26S rRNA genes in barley by *in situ* hybridization. *Genome* **35**, 1013-1018.
- LICHTER, P.; CREMER, T.; 1992: Non-isotopic *in situ* hybridization to metaphase chromosomes and interphase nuclei. EMBO Practical Course, EMBL, Heidelberg, Germany.
- MALUSZYNSKA, J.; HESLOP-HARRISON, J. S.; 1993: Physical mapping of rDNA loci in *Brassica* species. *Genome* **36**, 774-781.
- MARTIN, R.; BUSCH, W.; HERRMANN, R. G.; WANNER, G.; 1994: Efficient preparation of plant chromosomes for high-resolution scanning electron microscopy. *Chromosome Res.* **2**, 411-415.
- PATIL, V. P.; JADHAV, A. S.; 1985: Karyomorphology of three varieties of *Vitis vinifera*. *Cytologia* **50**, 83-88.
- PATIL, S. G.; PATIL, V. P.; 1992: Karyomorphology of *Vitis vinifera*, *V. rotundifolia* and their hybrid. *Cytologia* **57**, 91-95.
- REISCH, B. I.; LODHI, M. A.; YE, G. N.; WEEDEN, N. F.; 1994: A molecular marker-based linkage map of *Vitis*. VIth Int. Symp. Grape Breed., Yalta, Crimea, Ukraine.
- SCHMITT, T.; SCHWARZACHER, T.; HESLOP-HARRISON, J. S.; 1994: Physical mapping of rRNA genes by fluorescent *in situ* hybridization and structural analysis of 5S-rRNA genes and intergeneric spacer sequences in sugar beet (*Beta vulgaris*). *Theor. Appl. Genet.* **88**, 629-636.
- SUDHARSAN RAJ, A.; SEETHAIAH, L. 1973: Cytological studies in grape (*Vitis vinifera* L.). *Cytologia* **38**, 549-557.
- SHETTY, B. V.; 1959: Cytotaxonomical studies in Vitaceae. *Bibliogr. Genet.* **26**, 167-272.